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Acid and Base Resistance in *Escherichia coli* and *Shigella flexneri*: Role of *rpoS* and Growth pH

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***Escherichia coli* K-12 strains and *Shigella flexneri* grown to stationary phase can survive several hours at pH 2 to 3, which is considerably lower than the acid limit for growth (about pH 4.5). A 1.3-kb fragment cloned from *S. flexneri* conferred acid resistance on acid-sensitive *E. coli* HB101; sequence data identified the fragment as a homolog of *rpoS*, the growth phase-dependent sigma factor σ^{38}. The clone also conferred acid resistance on *S. flexneri rpoS*::Tn*10* but not on *Salmonella typhimurium*. *E. coli* and *S. flexneri* strains containing wild-type *rpoS* maintained greater internal pH in the face of a low external pH than strains lacking functional *rpoS*, but the ability to survive at low pH did not require maintenance of a high transmembrane pH difference. Aerobic stationary-phase cultures of *E. coli* MC4100 and *S. flexneri* 3136, grown initially at an external pH range of 5 to 8, were 100% acid resistant (surviving 2 h at pH 2.5). Aerobic log-phase cultures grown at pH 5.0 were acid resistant; survival decreased 10- to 100-fold as the pH of growth was increased to pH 8.0. Extended growth in log phase also decreased acid resistance substantially. Strains containing *rpoS*::Tn*10* showed partial acid resistance when grown at pH 5 to stationary phase; log-phase cultures showed <0.01% acid resistance. When grown anaerobically at low pH, however, the *rpoS*::Tn*10* strains were acid resistant. *E. coli* MC4100 also showed resistance at alkaline pH outside the growth range (base resistance). Significant base resistance was observed up to pH 10.2. Base resistance was diminished by *rpoS*::Tn*10* and by the presence of Na^+. Base resistance was increased by an order of magnitude for stationary-phase cultures grown in moderate base (pH 8) compared with those grown in moderate acid (pH 5). Anaerobic growth partly restored base resistance in cultures grown at pH 5 but not in those grown at pH 8. Thus, both acid resistance and base resistance show dependence on growth pH and are regulated by *rpoS* under certain conditions. For acid resistance, and in part for base resistance, the *rpoS* requirement can be overcome by anaerobic growth in moderate acid.**

The survival of bacteria under conditions in which growth is inhibited is a question of great current interest. *Escherichia coli* and *Shigella* species normally grow over the range of pH 5 to 9, for which internal pH is regulated at pH 7.4 to 7.9 during growth (16, 45, 49). Yet, cultures grown under certain conditions, such as stationary-phase (13) or log-phase growth in moderate acid (11), can remain viable following several hours of exposure at pH 2 to 3, well below the range permitting growth and cell division. Survival in acid may have clinical significance, because enteric pathogens must pass through the stomach at pH <3 for up to 2 h before colonizing the intestinal tract (10).

The survival of stationary-phase cells at extreme low pH is termed "acid resistance." As *E. coli* enters stationary phase, slowing and finally ceasing to grow, morphological and genetic changes occur to prolong survival and increase resistance to a variety of stress conditions (for review, see reference 40).

We report that the induction of stationary-phase acid resistance in *E. coli* and in *Shigella flexneri* requires σ^{38}, encoded by *rpoS* (*katF*), a major regulator of late-log-phase and stationary-phase growth (13, 25, 28, 40). Many gene products regulated by *rpoS* protect *E. coli* from adverse environmental conditions such as starvation, hyperosmolarity, oxidative damage, and UV radiation. An *rpoS* homolog in *Salmonella typhimurium* is required for chromosomal and plasmid-encoded virulence factors (5).

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Growth of log-phase cultures at a moderately low pH (pH 5.5 to 6.0) has also been shown to induce mechanisms of survival in more extreme acid, pH 2.5 (11). A similar phenomenon, termed "acid tolerance response," has been shown in *S. typhimurium* (6, 7). Acid tolerance correlates with improved pH homeostasis at low external pH; thus, acid induction of extreme acid tolerance may include inducible pH homeostasis (8). We measured the internal pH of wild-type and *rpoS* strains and tested the requirement for pH homeostasis in stationary-phase acid resistance.

We also tested the contribution of growth pH and anaerobiosis to acid resistance, because a number of genetic systems are known to be coinduced by acid and anaerobiosis (reviewed in reference 34). We found that growth pH has no effect on the stationary-phase acid resistance of wild-type cells, but acid-sensitive *rpoS*-defective strains show acid resistance following anaerobic growth in moderate acid. We also report the requirement of *rpoS* for survival at extreme alkaline pH (pH 9.8).

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used are identified in Table 1. Bacteria were grown in Luria-Bertani (LB) broth (30). Buffered LB broth contained a biological buffer of the appropriate pK_a for the given pH range (41), at 100 mM unless stated otherwise. Our buffers for various pH ranges are listed in reference 48. LB medium for extreme acid exposure (pH 1.5 to 3.0) was adjusted for pH with HCl, with no additional buffer. For extreme base exposure (pH 9.8 to 10.2), LB broth was buffered with 100 mM CAPS

TABLE 1. Bacteria strains and plasmids

Strain	Genotype	Source or reference(s)
Bacterial strains		
E. coli K-12		
HB101	*supE44 hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1* (*E. coli* K-12 × *E. coli* B hybrid)	3
MC4100	F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb5301 deoC1 ptsF24 rbsR*	2, 42
ZK1171	*rpoS*::Tn*10*	R. Kolter
JLS9300	MC4100 *rpoS*::Tn*10*	This study
HS122	*rpoS*::Mud (*lacZ* Km^r)	39
HS143	*rpoS*::Mud (*lacZ* Km^r)	39
UM122*katF*	*rpoS*(*katF*)::Tn*10*	32
S. flexneri		
3136		13
3136*rpoS*	3136 *rpoS*::Tn*10*	This work
S. typhimurium LB5000		C. Lee
Plasmids and phage		
pACYC184	Tet^r Cam^r	38
pREG153	Amp^r low-copy-number vector	23
pPS4.4	pACYC184 (*Cla*I fragment *S. flexneri rpoS*), Cam^r	This work
pPS1.3	pPS4.4 (*Eco*RV-*Hin*dIII fragment *rpoS*)	This work
pMMkatF3	*E. coli rpoS*(*katF*) Amp^r	32
λb221	*rex*::Tn*5*C1857 O_{am23} P_{am80}	A. Weiss

[3-(cyclohexylamino)-1-propanesulfonic acid], adjusted for pH with NaOH. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 10 μg/ml; kanamycin, 30 μg/ml; chloramphenicol, 30 μg/ml.

Log-phase cultures were diluted 1:100 from cultures grown overnight and were grown to an optical density at 600 nm (OD_{600}) of 0.1 at 37°C. Stationary-phase cultures were grown overnight at 37°C. Aerobic cultures at pH 5 or 8 and all anaerobic cultures were grown at least 24 h for stationary phase. Anaerobic cultures were grown under mineral oil with slow rotation.

Acid and base resistance. The definition of acid resistance was based on that of reference 13. Cultures were diluted 10^{-3} in LB broth (pH 2.5) and were incubated for 2 h at 25°C, unless stated otherwise. Some incubations were also done at 30°C, with similar results (data not shown.) Dilutions were plated on LB agar and grown overnight at 37°C, and colony counts were compared with those from plated dilutions of the original culture to determine the percentage of survival. At 100% survival, cell counts were generally 1×10^9 to 5×10^9/ml (stationary phase) or 1×10^8 to 5×10^8/ml (log phase). Values shown for percentage of survival represent the mean of at least six independent trials from overnight cultures from separate colonies. Base resistance was determined similarly, by diluting cultures 10^{-3} in LB broth–100 mM CAPS adjusted to pH 9.8 or 10.2 and incubation at 30°C, as indicated in the figures. Serial dilutions in buffered LB broth (pH 7.0) were plated and compared with dilutions from culture samples not treated with base.

***rpoS* phenotype.** The cell morphology phenotype of *rpoS* mutant strains was tested by light microscopy of cells in growth cultures grown in LB broth overnight (>20 h) (24, 40). Stationary-phase *rpoS* cultures produce more elongated and filamented cells than wild-type cultures. Catalase production was tested by adding a drop of 30% hydrogen peroxide to a bacterial colony and observing the evolution of bubbles (32).

Strain construction and β-galactosidase assay. *S. flexneri rpoS*::Tn*10* was constructed by P1 transduction from the donor strain *E. coli* UM122 (*rpoS*::Tn*10*) into *S. flexneri* 3136. For complementation studies, recombinant molecules containing acid resistance genes were electroporated into *E. coli* or *S. flexneri* with a Bio-Rad (Richmond, Calif.) electroporation apparatus according to company protocols.

E. coli rpoS::Tn*10* (strain JLS9300) was constructed by P1*vir* transduction from ZK1171 into MC4100, according to standard procedures (30, 42). The *rpoS* phenotype of this strain, JLS9300, was confirmed by observation of cell elongation and filamentation in stationary phase (24). The Tet^r element of Tn*10* inserted at non-acid resistance loci does not generally affect survival in acid (15).

lac fusion strains were assayed for β-galactosidase as described previously (30, 44).

Cloning strategy and procedures. A cosmid library of *S. flexneri* 3136 DNA was prepared by ligating a partial digest of *Sau*3A-cut DNA into a low-copy-number plasmid vector, pREG153, which had been cut with *Bam*HI and treated with alkaline phosphatase. Clones were packaged as lambda particles with a Giga-pak Plus packaging extract (Stratagene) and transfected into acid-sensitive *E. coli* HB101. An overnight growth of approximately 500 pooled cosmid clones was exposed to LB broth (pH 2.0) to select for acid-resistant clones, and a portion of the acid-treated cosmid mixture was plated on LB agar with ampicillin. Plasmid DNA was extracted from individual colonies of acid-resistant cosmid clones and digested with restriction endonucleases in order to identify a common fragment. Fragments of *Shigella* DNA from acid-resistant cosmids were subcloned into the plasmid vector pACYC184.

DNA sequence determination and protein analysis. The locus required for acid resistance in pPS4.4 was identified by Tn*5* mutagenesis with the delivery vector λb221. DNA sequence flanking the insertion site of Tn*5* in acid-sensitive mutants was obtaining with primers to IS50R. Sequencing was done with a Sequenase kit (U.S. Biochemical) according to the company's directions. The identities of the genes encoding acid resistance were obtained by submitting these sequences to the GenBank EMBL sequence data base for comparison with other known sequences. Both strands of the entire gene were then sequenced with CircumVent, a thermal cycle sequencing

kit purchased from New England Biolabs. Oligonucleotides for use as primers were synthesized on an Applied Biosystems 380B DNA synthesizer. Initially, primers for the upstream portion of the gene were made on the basis of the *E. coli* sequence (31, 32). However, we were unable to obtain sequence upstream of bp 400 by using this approach and therefore made all primers by moving up the *S. flexneri* sequence. DNA sequences were obtained for both strands of a 1.3-kb fragment containing the entire *rpoS* gene. The identification of cloned proteins was carried out with the Procaryotic DNA Directed Translation Kit made by Amersham. Protein was visualized by electrophoresis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (10% polyacrylamide) (38).

Measurement of internal pH. The transmembrane pH difference (ΔpH) was measured as follows on the basis of previously reported procedures (7, 48). For extreme acid-treated cells, a single colony was inoculated into a 250-ml flask containing 100 ml of LB broth, and the culture was shaken at 37°C overnight. The culture was diluted to an OD_{625} of 0.4 in 200 ml of LB broth and incubated at 30°C with gentle shaking for 2 h. Between pH 5.0 and 6.0, 100 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer was included. At pH 4.0, 20 mM citrate was used. For growth at pH 6.0, cultures were diluted 100-fold initially and grown exponentially up to an OD_{625} of 0.4. Where required, the internal pH of stationary-phase cells was decreased by the addition of 10 mM benzoic acid.

After incubation with or without growth (depending on external pH), the OD_{625} was remeasured. Twenty milliliters of culture was removed, and [^{14}C]salicylate or [^{14}C]benzoate was added (0.6 μM, 7 μCi/μmol) with ^3H_2O (80 μCi/ml) to label total cellular plus external volume. The flask was shaken at 30°C for 75 s. An aliquot was counted in a scintillation counter; the remainder was filtered through a Whatman GF/C filter under vacuum and then counted. The ΔpH was calculated on the basis of the Nernst equation, with intracellular volume determined as reported previously (48).

Nucleotide sequence accession number. DNA sequence data for both strands of the 1.3-kb fragment containing the *rpoS* gene mentioned above have been submitted to the GenBank-EMBL data base under accession no. UOO119.

RESULTS

Isolation of the *S. flexneri rpoS* homolog. We sought to identify cloned fragments of DNA from *S. flexneri* that confer acid resistance on *E. coli* HB101, a strain which is normally acid sensitive. Acid-resistant colonies of *E. coli* HB101 were isolated from a pool of *S. flexneri* cosmid clones exposed to pH 2.0 for 3 h. Out of 20 individual colonies assayed for acid resistance, 10 were fully acid resistant. Analysis with restriction endonucleases showed that all 10 acid-resistant cosmids shared a 4.4-kb *Cla*I fragment of *S. flexneri* DNA. This fragment was subcloned into the plasmid vector pACYC184 and designated HB101(pPS4.4). By using transposon mutagenesis, acid resistance was mapped to a 1.3-kb *Eco*RV-*Hin*dIII fragment within pPS4.4 (Fig. 1). This fragment was subcloned into pACYC184 and designated pPS1.3. Overlapping fragments of DNA flanking the insertion site of Tn*5* in four acid-sensitive mutants of HB101(pPS4.4) were sequenced and found to share over 90% identity with the *E. coli* gene *rpoS* (for review, see reference 14).

Upon sequencing the complete gene, an open reading frame of 1,088 bp encoding a protein of 362 amino acids was obtained. This gene shared 96% DNA identity and 97% amino acid identity with the sequence reported for *E. coli rpoS*(*katF*) (31). The promoter, ribosomal binding site, and termination sequences were homologous to those of *E. coli* (31, 46). The region upstream of the *S. flexneri* promoter, however, diverged significantly from the *E. coli* sequence in this area. The significance of these differences for *rpoS* regulation is being explored.

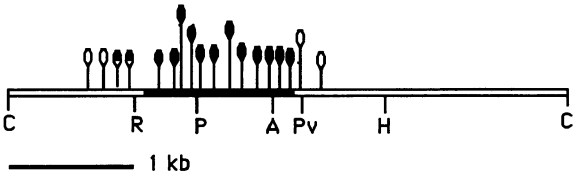

FIG. 1. Map of pPS4.4 showing locations of Tn*5* insertions. The coding region is shown in black. ●, insertions which result in the loss of acid resistance; ○, insertions which do not affect acid resistance; ◒, insertions which result in decreased acid resistance. A, *Acc*I; R, *Eco*RV; C, *Cla*I; H, *Hin*dIII; Pv, *Pvu*I.

In order to determine whether the *Shigella rpoS* homolog encoded a protein similar in size to that expected, plasmid DNA from HB101(pPS4.4), HB101(pPS1.3), and the acid-sensitive derivative HB101(pPS4.4::Tn*5*) was subjected to an in vitro transcription translation system. Both pPS4.4 and the subclone pPS1.3 yielded a translation product with a size of about 37 kDa. This product was lacking in pPS4.4::Tn*5-70* as well as in the plasmid vector pACYC184. The size of the protein expressed by our clones is in close agreement with that recently reported for σ^{38} from *E. coli* (46).

Complementation of acid sensitivity. In order to demonstrate the functional equivalence of *rpoS* in *S. flexneri* and *E. coli*, we constructed an *S. flexneri rpoS* mutant and conducted complementation studies (Table 2). *S. flexneri rpoS*::Tn*10* was found to be extremely acid sensitive. Acid resistance was restored to this strain by the introduction of either pPS4.4 or the *E. coli rpoS* clone pMMkatF3 (data not shown). The same clones also rendered HB101 fully acid resistant. On the basis

TABLE 2. Complementation of acid sensitivity in HB101, *S. flexneri* 3136 *rpoS*::Tn*10*, and *S. typhimurium* with cloned *rpoS*

Strain	% Inoculum surviving after 2 h in LB broth (pH 2.5)[a]
E. coli K-12	
HB101(pACYC184)	<0.001
HB101(pMMkatF3)[b]	80
HB101(pPS4.4)	82
HB101(pPS1.3)	50
S. flexneri	
3136	101
rpoS:Tn*10*	<0.001
rpoS:Tn*10*(pPS4.4)	100
rpoS::Tn*10*(pPS1.3)	10
S. typhimurium	
LB5000	<0.001
LB5000(pPS4.4)	<0.001
LB5000(pMMkatF3)	<0.001

[a] Data represent the mean of duplicate samples from a typical experiment. The inoculum was 10^4.

[b] pMMkatF3 contains a 4.2-kb fragment encompassing the *E. coli rpoS* gene cloned into the plasmid vector pAT153. This clone was provided by Peter Loewen, Department of Microbiology, University of Manitoba, Winnipeg, Canada.

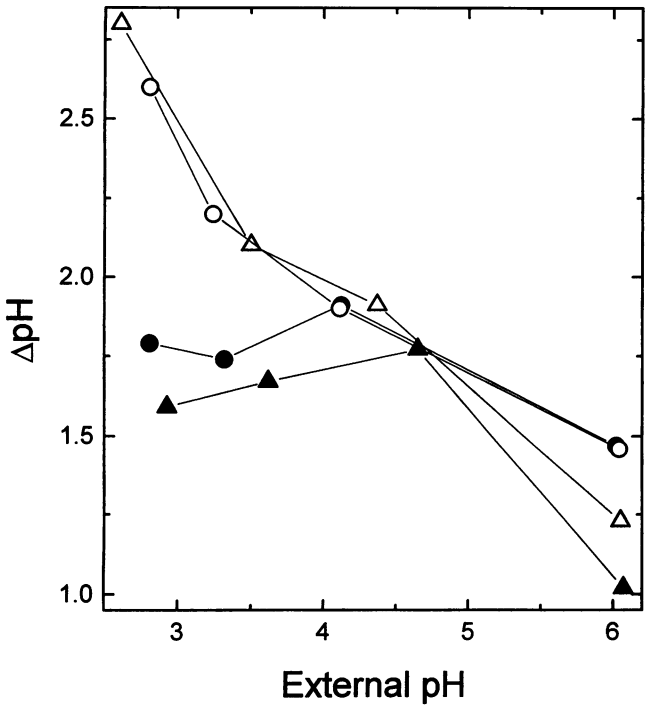

FIG. 2. ΔpH of wild-type and *rpoS* mutants of *S. flexneri* and *E. coli* K-12 at external pH 2.5 to 6.2. ○, *E. coli* HB101(pPS4.4); ●, *E. coli* HB101 (*rpoS* mutant); △, *S. flexneri* 3136; ▲, *S. flexneri* 3136 *rpoS*.

of morphological examination of stationary-phase cultures, HB101 appears to be an *rpoS* mutant. *Salmonella* species are extremely sensitive to acid under the conditions of our assay (13); however, transformation of *S. typhimurium* LB5000 by pPS4.4 or pMMkatF3 did not result in acid resistance (Table 2).

The pPS4.4 subclone pPS1.3, which contains the entire coding region of *rpoS* but lacks sequence upstream, only partially complemented acid resistance. Introduction of pPS1.3 into *S. flexneri rpoS*::Tn*10* restored acid resistance to only 1/10th the level of the parental strain, although complementation in *E. coli* HB101 was somewhat better (Table 2). The level of acid resistance found in HB101(pPS1.3) was still several orders of magnitude greater than that observed for HB101. Two clones containing Tn*5* insertions upstream of *rpoS* (Fig. 1) also showed partial complementation for acid resistance.

Several other phenotypes known to be regulated by *rpoS* in *E. coli* were tested in *S. flexneri* and in *S. flexneri rpoS*::Tn*10*. Hydroperoxidase (*katE*) activity (32), tested by observing the amount of bubbling upon addition of 30% hydrogen peroxide to colonies, was greatly diminished in the *rpoS* mutant. The *bolA*-dependent morphological change of stationary-phase cells (40) also was absent. We conclude that the *S. flexneri* gene is functionally equivalent to *E. coli rpoS*.

pH homeostasis under conditions of acid stress. The role of *rpoS* in pH homeostasis at extreme values of external pH was tested. Both in *S. flexneri* and in *E. coli*, *rpoS* contributed to pH homeostasis (Fig. 2) when the external pH fell below 4.0. At an external pH of 2.8, HB101(pPS4.4) containing the *S. flexneri rpoS* was able to maintain an internal pH of 5.4, whereas the internal pH of HB101 was only 4.6. For *S. flexneri*, the difference was even greater. Clearly, *rpoS* was required for optimal maintenance of pH homeostasis of stationary-phase cells exposed to extreme acid.

TABLE 3. Effect of benzoate on ΔpH and bacterial survival at external pH 3.3

Strain	Benzoate (10 mM)[a]	ΔpH	Internal pH	% Survival
E. coli MC4100	−	2.5	5.8	100
S. flexneri 3136	−	2.3	5.6	100
E. coli MC4100	+	1.6	4.9	40
S. flexneri 3136	+	1.6	4.9	28

[a] An overnight culture was diluted 10^{-3} in LB broth acidified with HCl to pH 3.3, with (+) or without (−) 10 mM benzoic acid as indicated. Incubation was for 2 h at 25°C.

The relationship between pH homeostasis and survival was tested. The membrane-permeant weak acid benzoate was added to cultures in order to conduct protons and depress internal pH. With the addition of 10 mM benzoate, the internal pH of *S. flexneri* decreased from 5.6 to 4.9 (Table 3). A slightly greater difference was found with *E. coli* MC4100. Despite the significant drop in internal pH, the acid resistance of benzoate-treated cultures was decreased only slightly compared with that of untreated cells. There was significant survival of both *S. flexneri* and *E. coli* after 2 h of exposure to pH 3.0 despite the fact that the internal pH of surviving cells is below 5.0.

Acid resistance in log phase: dependence on growth pH. In previous work, log-phase cultures of *S. flexneri* have been shown to be acid sensitive; the pH dependence has not been defined (13). The survival of *E. coli* and *S. flexneri* cultures incubated at pH 2.5 was tested as a function of growth phase and growth pH. Figure 3 shows that *E. coli* cultures grown at pH 5 to early log phase (OD_{600} of 0.1) show substantial acid resistance when the initial dilution of the stationary-phase overnight culture is 50- to 100-fold. At greater dilutions, the acid resistance of cells grown to the same OD_{600} falls off steadily; at 1,000-fold dilution, the level of acid resistance is comparable to that of an *rpoS*::Tn*10* strain tested in stationary

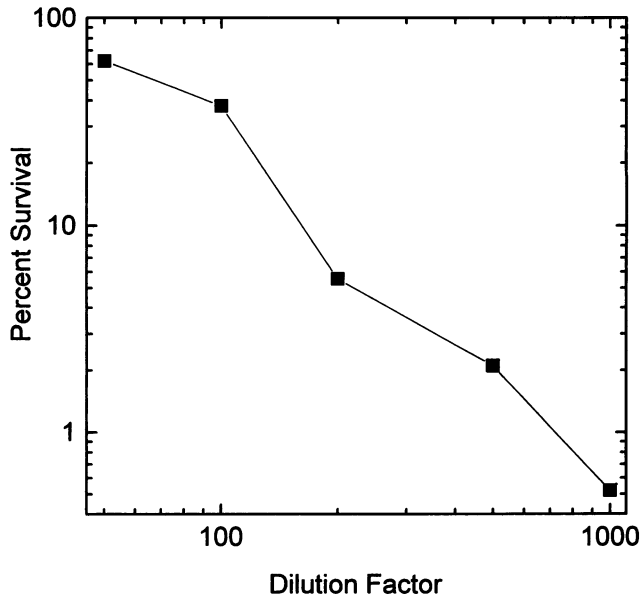

FIG. 3. Acid resistance in *E. coli* MC4100 as a function of extended log-phase growth. Cultures were grown overnight at pH 5 and then diluted as shown in buffered LB broth (pH 5) and grown to an OD_{600} of 0.1 before exposure to pH 2.5 for 2 h.

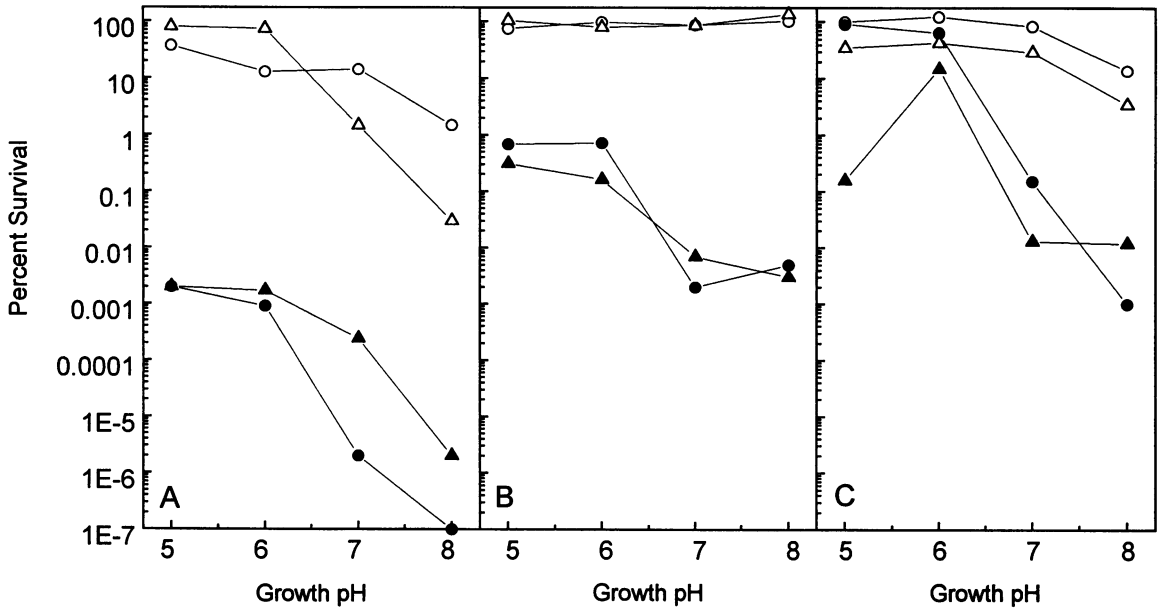

FIG. 4. Acid resistance in *E. coli* and *S. flexneri*. The percentage of survival of acid-treated cells (pH 2.5) compared with that of untreated cells is shown as a function of the pH of growth. Growth conditions were as follows: A, aerobic log phase (1:100 dilution); B, aerobic stationary phase; C, anaerobic stationary phase. ○, *E. coli* MC4100; ●, *E. coli* JLS9300 (*rpoS*); △, *S. flexneri* 3136; ▲, *S. flexneri* 3136 *rpoS*. *S. flexneri* 3136 was grown up to 40 h anaerobically.

phase (Fig. 4B). A similar effect of culture dilution was found for *S. flexneri* (data not shown.) These results suggest that some factor in stationary-phase cells persists over several generations of log-phase growth.

If the acid resistance of log-phase cells were solely a residual effect of previous stationary-phase growth, then the pH of log-phase growth would not be expected to affect acid resistance. To test this possibility, cells were grown aerobically to early log phase (from dilution 1:100 to OD_{600} of 0.1) at a range of external pH 5 to 8 (Fig. 4A). Log-phase cells grown at pH 5 showed 40 to 80% acid resistance, but as growth pH increased to pH 8, acid resistance decreased 10-fold (*E. coli*) and 1,000-fold (*S. flexneri*). Furthermore, this effect of pH during log-phase growth was independent of the pH of the overnight culture from which the initial dilution was made (data not shown). Thus, moderately low pH is required to sustain acid resistance, whereas growth at high pH turns off acid resistance early.

The presence of *rpoS*::Tn*10* decreased survival of acid-treated log-phase cultures by a factor of at least 10^{-4} (Fig. 4A). Interestingly, the pH dependence of acid resistance remained, even at these extremely reduced levels. We tested the possibility that pH-regulated expression of *rpoS* might play a role in log-phase acid resistance, although the pH dependence seen in the *rpoS*::Tn*10* strain made this unlikely. The induction of *rpoS* was determined by assay of *rpoS*::*lac* fusions (strains HS122 and HS143) (39). During early log-phase growth in buffered LB broth, these *rpoS*::*lac* strains showed 300 to 500 Miller units of activity with no significant dependence on growth pH (data not shown). Thus, pH-dependent expression of *rpoS* could not explain the growth pH dependence of log-phase acid tolerance.

Acid resistance in stationary phase: dependence on growth pH and anaerobiosis. Previous work suggests that stationary-phase acid resistance may be independent of growth pH (13). Aerobic stationary-phase cultures showed 100% acid resistance when grown at any pH, over the range of pH 5 to 8 (Fig. 4B). The survival of *rpoS*::Tn*10* strains of both *E. coli* and *S. flexneri* was substantially reduced, but not to the level seen in log-phase cultures (Fig. 4A). Acid-grown cultures of *rpoS*::Tn*10* strains showed close to 1% acid resistance; for comparison, acid-grown cultures of the *rpoS* mutant strain HB101 showed 5 to 10% extreme acid resistance.

Acidity and anaerobiosis are known to coregulate a number of cellular functions. Anaerobic cultures showed stationary-phase acid resistance comparable to that of aerobic cultures, with a slight drop-off in alkaline-grown cells (Fig. 4C). Anaerobic growth at pH 5 to 6 restored close to 100% acid resistance in the *E. coli rpoS*::Tn*10* strain. *S. flexneri rpoS*::Tn*10* showed partial recovery of acid resistance when grown anaerobically at pH 6; the decrease at pH 5 may be due to the relatively poor growth of *S. flexneri* at pH 5. These results showed that *rpoS* was not an absolute requirement for stationary-phase acid resistance, since the condition of anaerobic growth in acid largely compensated for the *rpoS* defect.

Base resistance in *E. coli*. Unpublished observations suggest that *rpoS* confers base resistance on *E. coli* (22). The ability of stationary-phase cultures of *E. coli* to survive exposure in alkaline media was tested (Fig. 5). The time course showed significant survival of the culture after up to 4 h of exposure at pH 9.8. The percentage of survival decreased sharply as the pH increased to 10.2. Thus, base resistance disappeared at a pH of less than 1 U beyond the limit of growth (pH 9). By contrast, acid survival was observed for several hours at pH 2, which is at least 2.5 U below the acid limit of growth.

The dependence of base resistance on growth pH and anaerobiosis is shown in Fig. 6. For these experiments, base resistance was defined as the percentage of cells surviving 2 h of incubation in buffered LB broth at pH 9.8. Base resistance was greatest in cultures grown at high pH (pH 8.0); cultures grown at pH 5 showed 10-fold less survival. The *rpoS*::Tn*10* strain showed 2 orders of magnitude less base resistance over

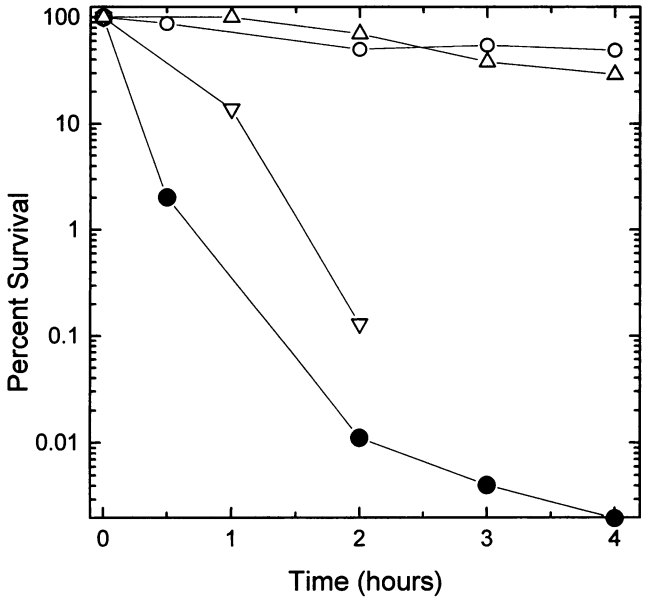

FIG. 5. Base resistance in *E. coli* as a function of exposure time. The percentage of survival of base- or acid-treated cells compared with that of untreated cells is shown as a function of time of exposure at 30°C. ○, MC4100 at pH 9.8; △, MC4100 at pH 2.0; ▽, MC4100 at pH 10.2; ●, JLS9300 (*rpoS*) at pH 9.8.

most of the pH range of growth. Surprisingly, a significant increase in base resistance was observed in *rpoS*::Tn*10* cultures grown anaerobically at lower pH. Thus, anaerobic growth in acid enhanced both acid resistance and base resistance of *rpoS*

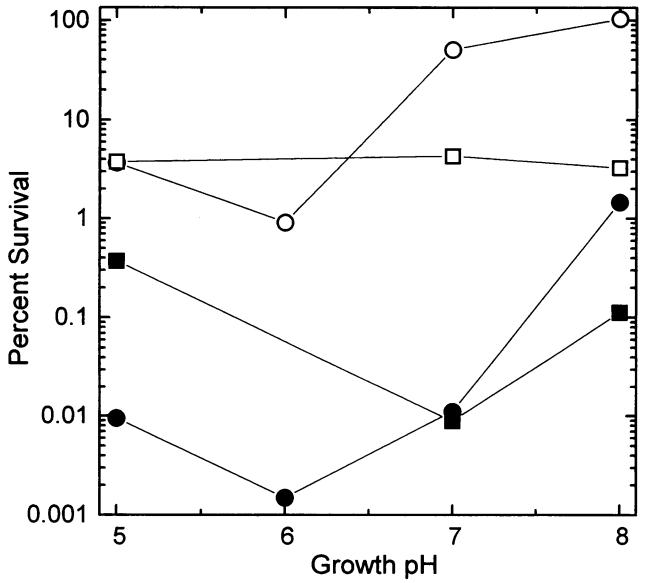

FIG. 6. Base resistance in *E. coli* as a function of pH of growth. The percentage of survival of base-treated cells (pH 9.8) compared with that of untreated cells is shown as a function of pH of growth. ○, *E. coli* MC4100, aerobic growth; □, *E. coli* MC4100, anaerobic growth; ●, *E. coli* JLS9300 (*rpoS*), aerobic growth; ■, *E. coli* JLS9300 (*rpoS*), anaerobic growth.

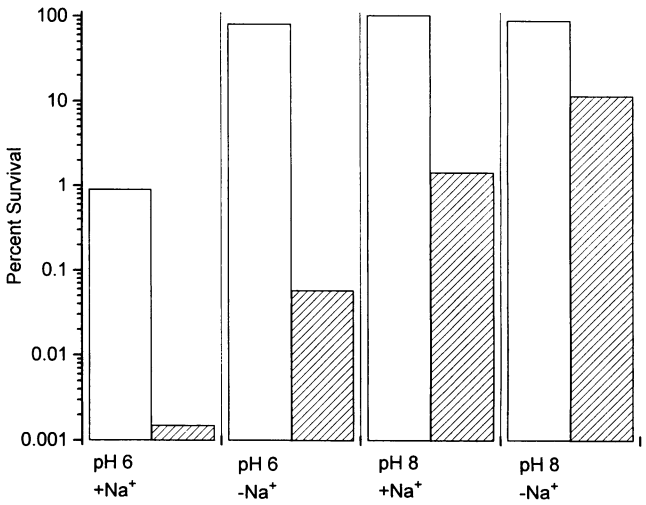

FIG. 7. Dependence of base resistance on Na^+ concentration. Open bars, MC4100; hatched bars, JLS9300. Cultures were grown overnight at pH 6 or 8 as indicated and then were diluted in buffered LB broth (pH 9.8) containing approximately 150 mM Na^+ ($+Na^+$) or excluding sodium other than nutrient contaminants ($-Na^+$). Cultures were incubated for 2 h at 30°C before plate assay.

mutant strains. Log-phase cultures showed <0.01% base resistance when grown at pH 5.0 or 8.0.

In *E. coli*, alkaline pH induces the Na^+/H^+ antiporter NhaA, which enhances growth at pH 8 in the presence of Na^+ (9, 17, 21). The effect of sodium ion on base resistance (pH 9.8) was tested. The inclusion of 200 mM NaCl at pH 9.8 decreased survival by 10^{-3}. Since buffered LB broth contains approximately 150 mM Na^+, the removal of sodium ion was tested by omission of NaCl from the LB recipe and adjustment of the pH of the buffer with KOH (Fig. 7); the residual Na^+ of LB broth was estimated at 10 mM (21). Cultures grown at pH 6 showed a 100-fold enhancement of base resistance in low-sodium media. Cultures grown at pH 8 survived at pH 9.8 with or without Na^+, but at pH 10.2, the elimination of Na^+ increased survival from 0.06% (regular buffered LB broth) to 50% (Na^+ replaced with K^+). Similar effects were also seen in the *rpoS*::Tn*10* strain. These results confirm the effect of Na^+ in survival at high pH above the growth range and suggest that the sodium effect is independent of *rpoS*.

S. flexneri showed relatively little base resistance compared with *E. coli*. Survival at pH 9.8 decreased rapidly over 2 h, even in low-sodium media (data not shown).

DISCUSSION

We have shown that *rpoS* is required for extreme acid resistance in aerobic cultures of *E. coli* and *S. flexneri* and for base resistance in *E. coli*. The degree of requirement for *rpoS* depends, however, on environmental factors (see below). The role of *rpoS* in log-phase acid resistance fits into a growing picture that *rpoS* has important functions during growth prior to stationary phase (33, 39, 46). Our results may resolve conflicting reports on the ability of *E. coli* to survive exposure to low acid. Early literature stated that an *E. coli* K-12 isolate was very acid sensitive (12), whereas a more recent examination of normal flora *E. coli* and several different K-12 strains has shown most *E. coli* strains to be very resistant to pHs below

3.0 (13). The fact that *rpoS* mutants arise frequently in various lineages of K-12 may explain these discrepancies (18, 19, 35).

The high degree of identity between the coding regions of *rpoS* in *S. flexneri* and *E. coli* is not surprising, considering the close taxonomic relationship of the two species. Sequencing of the complete *rpoS* gene from *S. typhimurium* has recently been completed (4), and like the *Shigella* gene, the *S. typhimurium* *rpoS* gene has been found to be highly homologous to *E. coli* *rpoS*. Nevertheless, the cloned *S. flexneri rpoS* did not complement acid sensitivity in *S. typhimurium* LB5000. This result shows that the relatively low level of acid resistance of *S. typhimurium* is probably not caused by a defect in *rpoS*. It is more likely that *Salmonella* spp. lack an *rpoS*-regulated gene or genes required for acid resistance.

The possibility that regulatory elements exist upstream from *rpoS* is suggested by the failure of the pPS1.3 subclone to fully complement *rpoS*::Tn*10*, as well as by the decrease in acid resistance found for two mutants containing transposon insertions which lie upstream of the *rpoS* open reading frame (Fig. 1). Since the greatest sequence divergence between *E. coli* and *S. flexneri* was observed in this region, the two species may exhibit some differences in regulation.

How do enteric organisms survive exposure to low pH? The presence of *rpoS* enhanced pH homeostasis at low external pH, a mechanism previously implicated in acid tolerance (8). Nevertheless, the depression of internal pH did not eliminate acid resistance. The addition of benzoic acid, a weak acid which freely diffuses into the cell but which has relatively little effect on electrical potential at the concentration and external pH used (37), lowered internal pH by almost a whole pH unit during extreme acid exposure (Table 3). Nonetheless, both *E. coli* and *S. flexneri* exhibited considerable survival despite the failure to maintain an internal pH above 5.4. By contrast, in studies by Foster et al., the survival rate of *S. typhimurium* is drastically reduced when the internal pH falls below 5.4 for 30 min (8). In Foster's studies, dinitrophenol was used to lower internal pH; this weak acid depresses both the transmembrane electrical potential as well as the ΔpH, a possible cause of the different results obtained. Alternatively, *S. typhimurium* may have a more sensitive requirement for maintenance of internal pH than does *E. coli*.

It is interesting that stationary-phase growth contributed to acid resistance even in the *rpoS*-defective strains (Fig. 4). Furthermore, some factor(s) of stationary phase must persist during early log-phase growth, because acid resistance persists over several generations in log phase. Growth at pH 8 appeared to turn off this factor. It may be that factors which enhance acid resistance are disadvantageous during alkaline growth. *rpoS*, however, contributed to both acid resistance and base resistance. We are in the process of identifying *rpoS*-regulated genes, as well as *rpoS*-independent genes, required for the ability to survive at low pH.

In interpreting the effects of growth phase, it is important to recognize that log phase and stationary phase are not well-separated categories. It is known that the expression of *rpoS* varies continuously across the range of growth from early-log phase to late-log phase (33). It may be best to think of the growth phase as a spectrum of states of the growing cell.

We also found evidence for two environmental factors which contribute to acid resistance: (i) acid pH during growth and (ii) anaerobiosis, both of which assisted acid resistance significantly even in the absence of *rpoS* (Fig. 4). It is particularly striking that the condition of moderate acid growth under anaerobiosis enabled substantial acid resistance for the *rpoS* strains and even conferred partial base resistance on *E. coli*. A growing number of genetic systems are known to be coinduced by acid and anaerobiosis, for example the *cadAB* operon in *E. coli* (29, 47), the fermentative lactate dehydrogenase *ldl* (*ldh*) (27), and, in *S. typhimurium*, the periplasmic protein *aniG* (1, 20).

Among closely related enteric bacterial species, similarities and differences in acid resistance appeared. *E. coli* MC4100 and *S. flexneri* showed generally similar responses to acid treatment (Fig. 4). In *S. typhimurium*, stationary-phase acid resistance has been seen (26), but the degree of resistance is considerably lower; cultures grown at pH 7.0 and exposed for 1 h to pH 3.0 show 1% survival. *rpoS* may not be required. Low pH of growth increases stationary-phase resistance in *Salmonella* spp. as it does for *rpoS* strains of *E. coli*. Thus, there appear to be significant differences between the regulatory circuits of *Salmonella* spp. and those of *E. coli*. The role of anaerobiosis in *Salmonella* acid resistance has not been elucidated.

Base resistance in *E. coli* showed some interesting parallels with acid resistance, as well as some differences. The protection conferred was less extreme, to alkaline levels barely 1 pH unit above the growth limit. This is not surprising, given the limited exposure to base in the intestine compared with the extreme acidity of the stomach. The contribution of *rpoS* was similar to that in acid resistance, suggesting that some aspects of response to extreme acid and base may share a common regulatory circuit. Log-phase base resistance or tolerance was not observed. There is, however, evidence that the death rate at extremely high pH (pH 11) is decreased in cultures grown at high pH (36).

The effect of pH on base resistance in stationary-phase cultures (Fig. 6) was complementary to the pH dependence for log-phase acid resistance; growth in a moderate base (pH 8) enhanced survival in a more extreme base (pH 9.8). The effect of anaerobiosis, however, was to enhance base resistance in acid-grown cells. Thus, even base resistance provides yet another connection between acid and anaerobic regulation (see reference 34 for review of pH-regulated genes, including anaerobic acid regulation).

The presence of Na^+ decreased the base resistance of *E. coli* (Fig. 7). Sodium sensitivity at high pH would be consistent with the well-documented requirement of the base-inducible sodium-proton antiporter NhaA to excrete sodium (9, 17, 21). *S. flexneri* showed both poorer growth at pH 8 and lower base resistance than did *E. coli*; the reason for this difference is not known. For *Salmonella* spp., base resistance has not been studied.

The finding of base resistance may have clinical significance, given that enteric bacteria are not only exposed to the strongly acidic stomach contents (pH 1 to 2) but are also exposed to the alkaline secretions (pH 9 or higher) from the pancreatic duct at the top of the small intestine, just a few centimeters below the pylorus. It may be conjectured that a regulatory mechanism which turns on both extreme acid resistance and base resistance could offer a selective advantage to cells exposed to acid followed shortly by base.

We are investigating further the components required for pH-dependent base resistance. We are also investigating the genetic requirements for *rpoS*-independent acid resistance in anaerobic acid-grown cultures and the connections between acid and base resistance.

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